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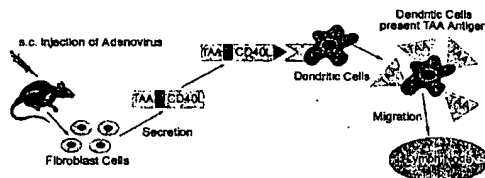
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(54) Title: ADENOVIRAL VECTOR VACCINE



(57) Abstract: Provided are adenoviral vectors for generating an immune response to antigen. The vectors comprise a transcription unit encoding a secretable polypeptide, the polypeptide comprising a secretory signal sequence upstream of a tumor antigen upstream of CD40 ligand, which is missing all or substantially all of the transmembrane domain rendering CD40L secretable. Also provided are methods of generating an immune response against cells expressing a tumor antigen by administering an effective amount of the invention vector. Further provided are methods of generating an immune response against cancer expressing a tumor antigen in an individual by administering an effective amount of the invention vector. Still further provided are methods of generating immunity to infection by human papilloma virus (HPV) by administering an effective amount of the invention vector which encodes the E6 or E7 protein of HPV. The immunity generated is long term.

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WO 2004/044176 A2

WO 2004/044176

PCT/US2003/036237

ADENOVIRAL VECTOR VACCINE

FIELD OF THE INVENTION

5 [0001] The present invention relates to the development of immunity against antigen expressing cells using an adenoviral vector that expresses the antigen fused to a secretable form of CD40 ligand.

BACKGROUND OF THE INVENTION

[0002] The following discussion of the background of the invention is merely
10 provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

[0003] The activation of antigen presenting cells (APCs) including the dendritic cells (DCs), followed by loading of the antigen presenting cell with relevant antigens, is a requisite step in the generation of a T cell dependent immune response against cancer
15 cells. Once activated and loaded with tumor antigens, DCs migrate to regional lymph nodes (LNs) to present antigens (ags) to T cells. Very commonly, these APCs express insufficient amounts of surface activation molecules (1) which are required for optimal activation and expansion of T cell clones competent to recognize tumor antigens. Antigen (ag) presentation to naive T cells, in the absence of costimulatory molecule
20 expression on the surface of the APC, leads to anergy of T cells (2). Moreover, cross-presentation by DCs without CD4⁺ T cell help also results in peripheral deletion of Ag-specific T cells in regional LNs (3). In contrast, in the presence of CD4⁺ T cell help, DCs change their functional ability to cross-prime T cells, resulting in clonal expansion of effector T cells (4). This CD4⁺ T cell help can be replaced with CD40-CD40 ligand
25 (CD40L) interactions (5). CD40L is a 33-kDa type II membrane protein and a member of the TNF gene family which is transiently expressed on CD4⁺ T cells after TCR engagement (6).

[0004] The ability of DCs to generate anti-tumor immune responses *in vivo* has been documented in a number of animal tumour models (7, 8). However, DC-mediated
30 induction of immunity represents a major therapeutic challenge. The current procedures

WO 2004/044176

PCT/US2003/036237

used for isolating and activating DCs are resource intensive and are difficult to apply to routine clinical practice. In addition, it is difficult to ensure that the antigen presenting cells express appropriate adhesion molecules and chemokine receptors to attract the these DCs to secondary lymphoid organs for priming T cells (9-14).

5

SUMMARY OF THE INVENTION

[0005] In one aspect, the present invention provides an adenoviral expression vector for generating immunity against antigen. The vector comprises a transcription unit encoding a polypeptide comprising, from the amino terminus, a secretory signal
10 sequence upstream of a tumor antigen upstream of CD40 ligand, which is missing all or substantially all of the transmembrane domain, rendering CD40 ligand secretable. The secretory signal sequence functions to direct the tumor antigen-CD40 ligand fusion protein to compartments of the cell which cause the fusion protein to be secreted from the cell.

15 [0006] In one embodiment, the tumor antigen is a human tumor antigen. In another embodiment, the human tumor antigen is the E7 protein of human papilloma virus (HPV).

[0007] In some embodiments, the transcription unit includes sequence that encodes a linker between the tumor antigen and the CD40 ligand. Suitable linkers may vary in
20 length and composition.

[0008] In another embodiment, the adenoviral expression vector comprises a human cytomegalovirus promoter/enhancer for controlling transcription of the transcription unit.

[0009] In still another embodiment, the CD40 ligand is human CD40 ligand.

25 [0010] In yet another embodiment, the CD40 ligand which lacks a functional transmembrane domain is one which contains less than 10% of the transmembrane domain or does not contain a transmembrane domain.

[0011] In another aspect, the present invention provides methods of generating an immune response in an individual against cells expressing a tumor antigen by

WO 2004/044176

PCT/US2003/036237

administering an effective amount of the invention vector which encodes the tumor antigen.

[0012] In one embodiment, the tumor antigen is a human tumor antigen. In another embodiment, the human tumor antigen is the E7 protein of human papilloma virus.

5 [0013] In yet another embodiment, the cells are cancer cells. In another embodiment, the cancer cells are cervical cancer cells.

[0014] In still another embodiment, the method results in the generation of cytotoxic CD8⁺ T cells against said tumor associated antigen. In another embodiment, the vector following administration is taken up by cells which subsequently secrete a fusion
10 protein encoded by the transcription unit.

[0015] In yet another aspect, the present invention provides methods of treating an individual with cancer that expresses a tumor antigen. The method comprises administering to the individual an effective amount of the invention expression vector which encodes the tumor antigen.

15 [0016] In one embodiment, the tumor antigen is a human tumor antigen. In another embodiment, the human tumor antigen is the E7 protein of human papilloma virus.

[0017] In yet another embodiment, the cells are cancer cells. In another embodiment, the cancer cells are cervical cancer cells.

[0018] In still another embodiment, the method results in the generation of cytotoxic
20 CD8⁺ T cells against said tumor associated antigen. In another embodiment, the vector following administration is taken up by cells which subsequently secrete a fusion protein encoded by the transcription unit.

[0019] In a further aspect, the present invention provides method of generating immunity in a subject to infection by human papilloma virus, comprising administering
25 to the individual an effective amount of the invention adenoviral expression vector wherein the tumor antigen is the E6 or E7 protein of human papilloma virus.

WO 2004/044176

PCT/US2003/036237

[0020] In the above methods, the vector is advantageously administered subcutaneously and may be given on subsequent time to increase the immune response. The immunity against the tumor antigen expressing cells is long lasting.

BRIEF DESCRIPTION OF THE DRAWINGS

5

[0021] FIG. 1 shows a model for events which follow subcutaneous injection of the secretable tumor antigen/CD40L vector. A proposal for the steps which occur following the injection of the subcutaneous space with respect to the activation, tumor antigen loading, migration to regional lymph nodes, activation of CD8 cytotoxic T cells which are specific for cells carrying the tumor antigen, and induction of a systemic T cell dependent Th1 like immune response.

10

[0022] FIG. 2 shows a scheme for constructing adenoviral expression vector encoding E7- Δ Ct Δ TmCD40L.

DETAILED DESCRIPTION OF THE INVENTION

15

[0023] In order to improve DC activation and tumor antigen loading, an immunization strategy was developed that used a recombinant adenovirus encoding a secretable form of the oncoprotein E7 from high-risk human papilloma viruses (HPV) fused to CD40 ligand from which the transmembrane domain had been deleted. This construct was engineered to be secretable by deleting the cytoplasmic and transmembrane domains from CD40 ligand, which was located downstream from E7 encoding DNA and DNA encoding a signal sequence.

20

[0024] The term "adenoviral expression vector" as used herein, refers to any adenoviral vector that includes exogenous DNA inserted into its genome which encodes a polypeptide. The vector must be capable of replicating and being packaged when any deficient essential genes are provided in trans. An adenoviral vector desirably contains at least a portion of each terminal repeat required to support the replication of the viral DNA, preferably at least about 90% of the full ITR sequence, and the DNA required to encapsidate the genome into a viral capsid. Many suitable adenoviral vectors have been described in the art. U.S. Patent no. 6,440,944; see 6,040,174 (replication defective E1

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WO 2004/044176

PCT/US2003/036237

deleted vectors and specialized packaging cell lines. A preferred adenoviral expression vector is one that is replication defective in normal cells.

[0025] The term "transcription unit" as it is used herein in connection with the adenoviral expression vector means a stretch of DNA that is transcribed as a single, continuous mRNA strand by RNA polymerase, and includes the signals for initiation and termination of transcription. For example, a transcription unit of the invention is nucleic acid that encodes from 5' to 3' a secretory signal sequence, the E7 protein from HPV and CD40 ligand without its transmembrane domain. The transcription unit is in operable linkage with transcriptional and/or translational expression control elements such as a promoter and optionally any upstream or downstream enhancer element(s). A useful promoter/enhancer is the cytomegalovirus (CMV) immediate-early promoter/enhancer (see U.S. Patents no. 5,849,522 and 6,218,140).

[0026] The term "secretory signal sequence" (aka. "signal sequence," "signal peptide," "leader sequence," or "leader peptide") as used herein refers to a short peptide sequence, generally hydrophobic in character, comprising about 20 to 30 amino acids which is synthesized at the N-terminus of a polypeptide and directs the polypeptide to the endoplasmic reticulum. The secretory signal sequence is generally cleaved upon translocation of the polypeptide into the endoplasmic reticulum. Eukaryotic secretory signal sequences are preferred for directing secretion of the exogenous gene product of the adenoviral expression vector. A variety of suitable such sequences are well known in the art and include the secretory signal sequence of human growth hormone, immunoglobulin kappa chain, and the like.

[0027] The term "tumor associated antigen" (TAA) as it is used herein refers to a protein which is present on tumor cells, and on normal cells during fetal life (onco-fetal antigens), after birth in selected organs, or on many normal cells, but at much lower concentration than on tumor cells. A variety of TAA have been described. In contrast, tumor specific antigen (TSA) (aka. "tumor-specific transplantation antigen or TSTA) refers to a protein not present on non-tumor cells. TSA usually appear when an infecting virus has caused the cell to become immortal and to express virus antigens. TSAs not induced by viruses can be idiotypes of the immunoglobulin on B cell lymphomas or the T cell receptor (TCR) on T cell lymphomas. Tumor-associated antigens (TAA) are more common than TSA.

WO 2004/044176

PCT/US2003/036237

[0028] Both TAA and TSA may be the immunological target of an adenoviral expression vector vaccine of the present invention. Unless indicated otherwise, the term "tumor antigen" is used herein to refer collectively to TAA and TSA. A preferred tumor antigen is the E6 or E7 protein of HPV. These antigens are preferably from HPV type

5 16.

[0029] The term "linker" as it used herein with respect to the transcription unit of the adenoviral vector refers to one or more amino acid residues between the carboxy terminal end of the tumor antigen and the amino terminal end of the CD40 ligand (lacking a functional transmembrane domain). The composition and length of the linker

10 may be determined in accordance with methods well known in the art and may be tested for efficacy. The linker is generally from about 3 to about 15 amino acids long, more preferably about 5 to about 10 amino acids long, however, longer or shorter linkers may be used or the linker may be dispensed with entirely.

[0030] The term "CD40 ligand" as used herein refers to a type II membrane polypeptide having an extracellular or cytoplasmic domain at its N-terminus, a transmembrane region and an extracellular domain at its C-terminus. CD40L is a member of the tumor necrosis factor superfamily of molecules and carries the designation TNF5. Unless otherwise indicated the full length CD40L is designated herein as "CD40L," "wtCD40L" or "wtTmCD40L." The form of CD40L where the

15 cytoplasmic domain has been deleted is designated herein as " Δ CtCD40L." The form of CD40L where the transmembrane domain has been deleted is designated herein as " Δ TmCD40L." The form of CD40L where both the cytoplasmic and transmembrane domains have been deleted is designated herein as " Δ Ct Δ TmCD40L." The nucleotide and amino acid sequence of CD40L from mouse and human is well known in the art and

20 can be found, for example, in U.S. Patent No. 5,962,406 (Armitage et al.).

25

[0031] Murine CD40L (mCD40L) is 260 amino acids in length. The cytoplasmic (Ct) domain of mCD40L extends approximately from position 1-22, the transmembrane domain extends approximately from position 23-46, while the extracellular domain extends approximately from position 47-260.

[0032] Human CD40L (hCD40L) is 261 amino acids in length. The cytoplasmic domain of hCD40L extends approximately from position 1-22, the transmembrane

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WO 2004/044176

PCT/US2003/036237

domain extends approximately from position 23-46, while the extracellular domain extends approximately from position 47-261.

[0033] The phrase "CD40 ligand is missing all or substantially all of the transmembrane domain rendering CD40 ligand secretable" as it is used herein refers to a recombina-
5 tant form of CD40 ligand that can be secreted from a cell. The transmembrane domain of CD40L containing about 24 amino acids in length functions to anchor CD40 ligand in the cell membrane. CD40L from which all of the transmembrane domain has been deleted is CD40 ligand lacking residues 23-46. CD40 ligand missing substantially all of the transmembrane is one that contains 6 residues or less of sequence at one end of
10 the transmembrane domain, more preferably less than about 4 residues of sequence at one end of the transmembrane domain, even more preferably less than about 2 residues of sequence on one end of the transmembrane domain, and most preferably 1 residue or less on one end of the transmembrane domain. Thus, a CD40L that lacks substantially all of the transmembrane domain rendering the CD40L secretable is one that contains no
15 more than six residues of sequence on one end of the domain. Such as CD40L would contain, in addition to the extracellular domain and optionally the cytoplasmic domain, no more than amino acids 41-46 or 23-28 located in the transmembrane domain of CD40L.

[0034] It should be understood that a CD40L which lacks a functional
20 transmembrane domain may still include all or a portion of the cytoplasmic domain. Likewise, a CD40L which lacks a functional transmembrane domain may include all or a substantial portion of the extracellular domain.

[0035] As used herein, an adenoviral vector of the present invention can be administered as a vaccine to induce immunity to a tumor associated antigen. The viral
25 vector may be formulated as appropriate with a pharmaceutically acceptable carrier. Accordingly, the viral vectors may be used in the manufacture of a medicament or pharmaceutical composition. Viral vectors of the invention may be formulated as solutions or lyophilized powders for parenteral administration. Powders may be reconstituted by addition of a suitable diluent or other pharmaceutically acceptable
30 carrier prior to use. Liquid formulations may be buffered, isotonic, aqueous solutions. Powders also may be sprayed in dry form. Examples of suitable diluents are normal isotonic saline solution, standard 5% dextrose in water, or buffered sodium or

WO 2004/044176

PCT/US2003/036237

ammonium acetate solution. Such formulations are especially suitable for parenteral administration, but may also be used for oral administration or contained in a metered dose inhaler or nebulizer for insufflation. It may be desirable to add excipients such as polyvinylpyrrolidone, gelatin, hydroxy cellulose, acacia, polyethylene glycol, mannitol,
5 sodium chloride, sodium citrate, and the like.

[0036] Alternately, viral vectors may be prepared for oral administration. Pharmaceutically acceptable solid or liquid carriers may be added to enhance or stabilize the composition, or to facilitate preparation of the viral vectors. Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, magnesium stearate or
10 stearic acid, talc, pectin, acacia, agar or gelatin. Liquid carriers include syrup, peanut oil, olive oil, saline and water. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax. The amount of solid carrier varies but, preferably, will be between about 20 mg to about 1 g per dosage unit. When a liquid carrier is used, the preparation may be in the form of a
15 syrup, elixir, emulsion, or an aqueous or non-aqueous suspension.

[0037] Viral vectors of the invention may be formulated to include other medically useful drugs or biological agents. The viral vectors also may be administered in conjunction with the administration of other drugs or biological agents useful for the disease or condition that the invention compounds are directed.

20 [0038] As employed herein, the phrase "an effective amount," refers to a dose sufficient to provide concentrations high enough to generate an immune response in the recipient thereof. The specific effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated, the severity of the disorder, the activity of the specific compound, the route of administration, the rate of
25 clearance of the viral vectors, the duration of treatment, the drugs used in combination or coincident with the viral vectors, the age, body weight, sex, diet, and general health of the subject, and like factors well known in the medical arts and sciences. Various general considerations taken into account in determining the "therapeutically effective amount" are known to those of skill in the art and are described, e.g., in Gilman et al.,
30 eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990. For administration of adenoviral vectors, the range

WO 2004/044176

PCT/US2003/036237

of particles per administration typically if from about 1×10^7 to 1×10^{11} , more preferably 1×10^8 to 5×10^{10} , and even more preferably 5×10^8 to 2×10^{10} . A viral vector can be administered parenterally, such as intravascularly, intravenously, intraarterially, intramuscularly, subcutaneously, or the like. Administration can also be orally, nasally, rectally, transdermally or inhalationally via an aerosol. The viral vectors may be administered as a bolus, or slowly infused. The vector is preferably administered subcutaneously.

[0039] The E7 protein derived from the human papillomaviruses (HPVs) was chosen as the exemplary TAA since it has been shown to be a strong stimulus of the cellular immune response, and to be expressed on every cell of a tumor population which is generated from HPV transformed tissue. HPV can cause a variety of epithelial lesions of the skin and genital tract. HPV related diseases of the genital tract constitute the second leading cause of cancer death among women in the world. These include genital warts, cervical intraepithelial neoplasia (CIN) and cancer of the cervix. The HPV type most commonly associated with high grade CIN and cervical cancer is HPV type 16. The majority of cervical cancers express the non-structural HPV16-derived gene products E6 and E7 oncoproteins. In HPV-induced cervical cancer model, the E6/E7 oncoproteins are required for maintenance of the malignant phenotype and their expression correlates with the transforming potential of HPV16(15-16). Therefore, E6 and E7 represent a target of choice for the therapeutic vaccination (17-19).

[0040] The results herein show that the subcutaneous injection of this vector leads to; 1) Local infection of cells surrounding the needle tract; 2) Secretion of the chimeric E7/CD40L transmembrane less fusion protein over a 10 day period from the vector injected cells; 3) Binding of the fusion protein to APCs leading to activation of the APC's and E7 presentation; 4) Migration of the loaded and activated APCs to regional lymph nodes, and 5) Evolution of T cell dependent systemic immunity to cell lines carrying the tumor associated antigen which extends for greater than one year.

[0041] Generation of cytotoxic T cell lymphocytes specific for individual populations of tumor cells by antigen presenting cells (dendritic cells) depends on the activation of expression of co-stimulatory molecules. One of the mechanisms through which this can occur is the binding of the CD40 ligand on the plasma membrane of CD4 Helper T Cell lymphocytes to the CD40 receptor on the dendritic cells. Following this

WO 2004/044176

PCT/US2003/036237

activation, dendritic cells migrate to region lymph nodes or to the spleen in which the presentation of the peptides derived from tumor associated antigens induces the activation and expansion of CD8 cytotoxic T cell lymphocyte clones which are competent to recognize the tumor associated antigen.

- 5 [0042] The evolution of such CD8 cytotoxic T cell lymphocytes, which can recognize tumor associated antigens on tumor cells, which leads to the killing of the tumor cells bearing these antigens, is limited by many factors. One factor is the low level of transfer of the tumor associated antigens from tumor cells into the antigen presenting cells. Another is the failure to induce the co-stimulatory molecules on the
10 antigen presenting cells which are necessary for sustained activation and expansion of tumor antigen specific CD8 cytotoxic T cell lymphocytes which are competent to recognize and kill the tumor cells.

- [0043] Antibodies which bind to the CD40 receptor on antigen presenting cells can activate the expression of the co-stimulatory molecules in the antigen presenting cells.
15 *In vitro* infection of the antigen presenting cells (either dendritic cells or tumor cells themselves) with an adenoviral vector (Ad-wtTmCD40L) which carries a CD40 ligand transcription unit has also been used to activate co-stimulatory molecule expression on the antigen presenting cells or the tumor cells (crystal). Injection of these dendritic cells into the tumor nodules or injection of the irradiated Ad-wtTmCD40L infected tumor
20 cells into immunocompetent mouse hosts carrying tumor nodules can lead to regression of the tumor nodules and to increased survival.

- [0044] In addition, *in vivo* generation of tumor antigen specific CD8 cytotoxic T cell lymphocytes results from *in vitro* "loading" of the antigen presenting cells with tumor associated antigens by incubation with tumor antigen peptide fragments, or with the
25 tumor cells themselves, or with adenoviral vectors which carry transcription units encoding the tumor associated antigens, followed by injection of the antigen loaded antigen presenting cells.

- [0045] In order to develop a more efficient way of generating a tumor antigen CD8 cytotoxic T cell lymphocyte dependent systemic immune response to tumor associated
30 antigens, it was hypothesized that the continuous release of a protein in the subcutaneous space of immunocompetent animals, which was capable of both activating

WO 2004/044176

PCT/US2003/036237

the expression of co-stimulatory molecules in antigen presenting cells and at the same time delivering tumor associated antigen into the intracellular space of antigen presenting cells over a 10 day period, could lead to a very strong CD8 cytotoxic T cell lymphocyte response against the tumor cells bearing the tumor associated antigen.

- 5 [0046] The fusion protein transcription unit that was chosen to test this hypothesis comprised a tumor associated antigen (the human papilloma virus E7 transforming protein) linked to the amino-terminal end of the CD40 ligand. This protein was engineered to be secreted from cells in which it was being made by deleting the transmembrane and cytoplasmic domains of the CD40 ligand, and attaching a human
- 10 growth hormone signaling domain to the amino-terminal end of the E7 tumor antigen portion of the fusion protein gene. Subcutaneous injection of an adenoviral vector which carried a transcription unit encoding this secretory form of the tumor associated antigen/CD40 ligand fusion protein (Ad E7- Δ Ct Δ TmCD40L) was chosen to generate adenoviral infected cells in the subcutaneous space of a subject.
- 15 [0047] It was reasoned that the cells infected in the vicinity of the site of subcutaneous injection of the vector, would release the tumor antigen/CD40 ligand secretory protein for up to 10-14 days (the time period during which most adenoviral vector infected cells produce the protein product of the transgene). Antigen presenting cells (e.g. DC) in the vicinity of the infected cells at the injection site would take up E7
- 20 tumor associated antigen, which would be digested in the proteosome with the resultant E7 peptides trafficking to the endoplasmic reticulum where they would bind to Class I MHC molecules. Eventually, the DCs would present the E7 tumor associated antigen on the surface in the Class I MHC molecule. Activated, tumor antigen-loaded antigen presenting cells would migrate to lymphocyte bearing secondary organs such as the
- 25 regional lymph nodes or the spleen. During the two weeks of continuous release of the tumor antigen/CD40 ligand fusion secretory protein, CD8 cytotoxic T cell lymphocytes which were competent to recognize and kill cells, which carried the tumor associated antigens, would be expanded in the lymph nodes and spleen by the presence of the activated and antigen loaded dendritic cells. The continuous nature of the stimulation
- 30 and the expansion of the tumor antigen specific cytotoxic T cells by the continuous release from the vector infected cells was believed to generate an immune response which would be greater in magnitude than were possible using a vector which carried a

WO 2004/044176

PCT/US2003/036237

tumor associated antigen/CD40 ligand which was non-secretory. An outline of the events that would occur during the release of secretable form of the E7/CD40L are shown in FIG. 1. The constant release of the fusion protein and the resultant continuous entry of activated DCs bearing the E7 peptides into the regional lymph nodes was
5 believed to provide an more effective *in vivo* way to generate a strong and durable T cell dependent systemic immune response against E7 positive tumor cells than injection of a vector which expresses E7 alone or CD40L alone, or a vector that expresses E7/CD40 ligand transcription where CD40L is not secretable.

[0048] The results of the experiments described herein were designed to test these
10 hypotheses and confirmed all the predictions. Subcutaneous injection of a replication incompetent adenoviral vector, which carries a secretory form of the E7 tumor associated antigen/CD40 ligand protein, induced a very robust and long lasting CD8 cytotoxic T cell lymphocyte dependent systemic immune response against cancer cells which carry the E7 tumor associated antigen. Indeed, the experimental results showed
15 that the vector expresses the secretory form of the tumor associated antigen/CD40 ligand fusion protein generated an immune response which is much stronger than the subcutaneous injection of a vector that expresses the non-secretory form of the E7 tumor associated antigen/CD40 ligand fusion protein. It is clear from the these experimental results that the subcutaneous injection of vectors encoding the tumor associated
20 antigen/CD40 ligand secretory fusion protein produces an immune response that is much stronger than occurs following the subcutaneous injection of the vector which carries transcription units encoding only for the E7 tumor associated antigen or the CD40 ligand (secretory or non-secretory).

[0049] In addition, it was shown that the local release of the fusion protein generates
25 activation of antigen presenting cells, and their migration to the lymphoid bearing secondary organs. Finally, it was demonstrated herein that the immunological resistance generated by the subcutaneous injection of adenoviral vectors carrying the E7 tumor associated antigen/CD40 ligand secretory transcription unit was specific to cells carrying the tumor associated antigen and was long lasting for up to one year after the
30 vector injection. Although not wishing to be bound by any theory, it is believed that the invention method of linking the tumor associated antigen to a secreted form of CD40 ligand creates a protein which can at the same time both activate antigen presenting cells

WO 2004/044176

PCT/US2003/036237

and antigen load them *in vivo* avoiding the expense and the logistical problems associated with *in vitro* generation of antigen presenting cells or cytotoxic T cell lymphocytes.

5 [0050] A second advantage is that the use of the secreted tumor associated antigen/ CD40 ligand fusion protein for activation and antigen loading may result in selective binding to the antigen presenting cells and transport of the antigen into the intracellular space of the antigen presenting cell. This would solve the problem of delivering sufficient tumor antigens into the intracellular space of a subject which is frequented by antigen presenting cells so as a sufficient density of tumor associated peptide on the
10 plasma membrane of the antigen presenting cells for generating a strong cytotoxic T cell lymphocyte response against the tumor associated antigen expressing tumor cell.

[0051] A third advantage is that the presence of the CD40 ligand in the secreted protein may activate co-stimulatory antigen expression on the antigen presenting cell, thus making the activation independent of initial CD4 T cell lymphocyte help.

15 [0052] A fourth advantage of the secretory nature of the viral fusion protein transcription unit is that there may be continuous release of the activating and antigen loading fusion protein in the space populated by antigen presenting cells over a 10 day period. This may account in part for the robustness of the immune response observed and its durability.

20 [0053] The decision to utilize the HPV E7 protein as the tumor associated antigen of the fusion protein was motivated by several factors. The first is that the E7 protein has been shown to be transforming and essential for the continued proliferation of HPV transformed cells. This property of E7 ensures that most if not all of the cells in the population would carry the antigen. In addition, the choice of a DNA virus
25 transforming protein as the tumor associated antigen was designed to make the experiments unambiguous with respect to the testing of the value of the secretory fusion transcription unit. A separate but important reason for selecting HPV E7 as the tumor associated antigen in the invention vector was to evaluate the ability of E7 to prevent the infection and proliferation of HPV infected cervical epithelial. If such vector could
30 generate an immune response against HPV infected cells, then individuals in whom such

WO 2004/044176

PCT/US2003/036237

a reaction would be generated would be immune and resistant to the infection of the human papilloma virus.

[0054] Human papillomavirus (HPV) infection induces a natural host immune response that is not sufficient to eliminate infection. One reason for this is that there are
5 over 100 genotypes of HPV each of which may have a unique amino acid sequence and therefore unique epitopes that allow the virus to escape detection by individuals exposed to other HPV genotypes. A second reason is that there are a small number of individuals who are unable to mount a cellular immune response to HPV infected cells. Such individuals may be the ones in whom chronic HPV infections develop which lead
10 to cervical dysplasia and to invasive cervical cancer. In addition, 60% of human cervical cancers are related to infections with the HPV 16 genotype. Up to 95% of cervical cancer cases are attributable to HPV infections. The second leading cause of cancer death among women worldwide is HPV associated malignancies. In emerging nations of the world, HPV associated malignancies represent the leading cause of death
15 among women between the ages of 25 and 35.

[0055] In order to amplify an immune response to the HPV infected cells so as to develop immunity to HPV infection, several methods have been attempted. The therapeutic peptide and protein-based vaccines (15), DNA (16) and viral vector-based vaccines (17), modified tumor cell (18) and dendritic cell-based vaccines (19) all have
20 been previously investigated and reported to generate immune responses to human HPV-associated neoplasms in animal models. These previous studies have shown that a variety of preparation of DCs can stimulate an effective antitumor immunity, including DCs loaded with proteins, DCs fused with tumor cells and DCs transduced with tumor-derived RNA or viral vectors (20,21,22). At present, *in vitro* CD40 ligand activation of
25 MART-1 gene modified DC can promote CD8 T cell lymphocyte mediated immunity to melanoma cells (23). Injection of CD40 ligand-transduced tumor showed a therapeutic effect against the established MCA205 brain tumor cell line (24). These studies indicated that DCs that were loaded with antigen and activated at the same time can provide strong protective immunity.

30 [0056] Recombinant adenoviral vectors offer a potentially superior approach that allows a higher efficiency of gene transfer than that of DNA vaccines. Adenoviral vectors encoding tumor associated antigens also can induce the protective cellular and

WO 2004/044176

PCT/US2003/036237

humoral immunity against such antigens, including those to which tolerance had developed.

[0057] Replication-defective adenoviral vectors, used herein to generate continual local release of a fusion protein composed of a tumor antigen (E7) linked to a secretory form of CD40 ligand, facilitated DCs maturation, promoting the development of effective antigen-specific immunity. It was demonstrated that secretable E7- Δ Ct Δ TmCD40L construction dramatically enhances the potency of the cellular immune response to E7 positive tumor cells. Subcutaneous injection of the Ad E7- Δ Ct Δ TmCD40L vector elicited strong E7-specific CD8⁺ T cell-mediated immunity, which could prevent the engraftment of cancer cells which express the E7 tumor associated antigen.

[0058] Although not wishing to be bound by any theory, a potential mechanism for the observed enhancement of E7-specific CD8⁺ T-cell activity following injection of the vector encoding the tumor antigen/secretable CD40L is the enhanced activation and migration of dermal professional APCs which are generated by the subcutaneous release of fusion protein from vector infected cells. In contrast to the strategy of loading DCs with antigen by administering the antigen, secretion of the E7- Δ Ct Δ TmCD40L protein, by the dermal fibroblasts or other cells in the subcutaneous space may more effectively activate and load local APCs, which may be effective in activating systemic T-cell immunity.

[0059] Another factor potentially affecting enhancement of antigen-specific CD8⁺ T cell activity by the AdE7- Δ Ct Δ TmCD40L vector is the use of the full-length E7 protein as the tumor associated antigen. Some workers have compared immune responses achieved with vaccines based on either HPV cytolytic T lymphocyte (CTL) plus helper T lymphocyte epitopes or the CTL epitope alone derived from the E7 peptide of the HPV16 strain. The results showed that the E7-specific CD8⁺ response was significantly greater for the vaccine based on HPV epitopes plus helper Th epitopes than for the vaccine based only on the CTL epitope (15).

[0060] In summary, the findings illustrate that linkage of Δ TmCD40L, which is a secretory form of a CD40L, to a tumor associated antigen, can lead to enhanced antigen-specific CD8⁺ T-cell activity *in vivo*.

WO 2004/044176

PCT/US2003/036237

[0061] The following examples serve to illustrate the present invention. These examples are in no way intended to limit the scope of the invention.

EXAMPLES

1. Construction of adenoviral expression vectors

5 [0062] The transcription unit, E7- Δ Ct Δ TmCD40L, of the adenoviral vector encoded a signal secretory sequence followed by the HPV type 16 E7 gene which was connected via a linker to the fragment of the CD40 ligand which contained the extracellular domain without the transmembrane or cytoplasmic domains. E7 was small enough that it did not disrupt the natural assembly of a homotrimeric array trimeric of the native
10 wild type CD40 ligand. The fusion protein was engineered to be secreted from vector infected cells by the addition of a signal sequence to the amino-terminal end of the E7 protein and by deletion of the transmembrane and cytoplasmic domains of the CD40 ligand.

[0063] The transcription unit was introduced into the E1 gene region of the
15 adenoviral vector backbone. After the adenoviral vector particles were generated in HEK 293 cells, the vector DNA was purified by cesium chloride gradient centrifugation. The presence of the signal peptide in the adenoviral vector was confirmed by restriction enzyme analysis and by DNA sequencing.

[0064] Adenoviral vectors with different transcription units including Ad-E7-
20 Δ TmCD40L, Ad-E7-wtTmCD40L Ad- Δ TmCD40L, Ad-wtTmCD40L, and Ad-GFP- Δ TmCD40L were constructed (see materials and methods) (GFP refers to green fluorescent protein).

2. Cell free expression Adenoviral vector transcription units

[0065] An *in vitro* cell free transcription/translation system was used to confirm that
25 the adenoviral vector containing transcription unit encodes the correct molecular weight protein. The results summarized in Table I show that the proteins produced by the *in vitro* transcription/translation reactions using PCR-generated cDNAs as templates migrated as expected with the following molecular weights: the E7- Δ Ct Δ TmCD40L is 32 kDa, E7-wtCD40L is 39 kDa, and the Δ Ct Δ TmCD40L is 22 kDa.

WO 2004/044176

PCT/US2003/036237

3. Western blot analysis of the proteins in cells 293 infected by the recombinant Adenovirus

[0066] Western blot analysis was used to evaluate the proteins expressed in cells infected with the different adenoviral vectors. The results summarized in Table I show that molecular weights of the proteins produced in the vector infected cells from the Ad-E7- Δ Ct Δ TmCD40L vector, the Ad-wtTmCD40L vector, the Ad-GFP- Δ Ct Δ TmCD40L vector, the Ad-wtTmCD40L, and the Ad- Δ Ct Δ TmCD40L vectors are respectively: 32kDa, 35kDa, 50kDa, 39kDa and 22kDa. These data confirm that the transcription units are structurally correct and that the vector DNA in each case can be transcribed and translated into protein at sufficient levels to be detected in a Western blot.

Table I : Molecular weight of viral vector translation products

Vector	Molecular Weight Cell Free Transcription Translation	Molecular Weight by Western
Ad-E7- Δ Ct Δ TmCD40L	32 kDa	32 kDa
Ad-E7-CD40L	39 kDa	32 kDa
Ad- Δ Ct Δ TmCD40L	22 kDa	22 kDa
Ad-CD40L	37 kDa	37 kDa
Ad-GFP- Δ Ct Δ TmCD40L	Not Done	50 kDa

4. Comparison of the Adenoviral expression vectors for efficiency of activation of DCs.

[0067] The various adenoviral vectors were compared for efficiency of activation of DC cells. For this purpose, bone marrow derived DCs were exposed *in vitro* to each of the vectors, under conditions that result in infection of close to 100% of the test cells (293 cells). After 48 hours, the cells were reacted with antibodies to activation markers CD80 and CD54 and evaluated in a fluorescent activated cell sorter. The percentage of cells positive for the CD80 and CD54 activation markers on the DCs exposed to the vectors carrying the secretable CD40L transcription units (either the E7/CD40L or the CD40L) was far above that seen with the DCs exposed to the vectors carrying either of

WO 2004/044176

PCT/US2003/036237

the non-secretable CD40L transcription units (wtTmCD40L, or E7/wtTMCD40L), or the Ad-GFP vector as shown below in Table II.

Table II : Expression of Activation Markers on DC

	Percent CD 80+	Percent CD54+
AdE7-wtCD40L	15.27%	17.66%
AdE7- Δ TmCD40L	40.4%	38.6%
AdGFP	4.63%	3.87%

- 5 These results indicate that the adenoviral vector with the secretable CD40L transcription unit is much more efficient in activating DCs *in vitro* than the vectors carrying the non- secretable transcription units.

10 **5. Comparison of IL-12 and IFN- γ cytokine release from DCs infected by Adenoviral expression vectors.**

[0068] Activation of DCs as measured by the release of cytokines IL-12 and IFN-gamma was determined for the various adenoviral vector transcription units. A statistically significant difference in the level of induction of IL-12 production following exposure of the DCs to the Ad-E7- Δ Ct Δ TmCD40L and Ad-E7-CD40L vectors was
15 observed ($P < 0.0001$). Specifically, 18 ± 4 pg / 2×10^5 cell / ml/24h and 88 ± 29 pg/ 2×10^5 cell / ml/48h of IL-12 were produced by the DCs exposed to the AdE7- Δ Ct Δ TmCD40L vector, whereas exposure of DCs to the Ad-E7-CD40L vector, produced 0 pg / 2×10^5 cell / ml/24h and 7 pg/ 2×10^5 cell / ml/48h of IL-12.

20 [0069] Similarly, there was a statistically significant difference in the amount of IFN- γ released by DCs exposed to the Ad-E7- Δ Ct Δ TmCD40L vector (335 ± 29 pg) as compared to DCs exposed to the Ad-CD40L vector (186 ± 9 pg) in first 24 hours ($P < 0.0001$). However, there was no significant difference between the level of IL-12 produced by DCs exposed to the vectors containing the secretable vs the non-secretable
25 E7/CD40L transcription units at 48 hours.

[0070] These data indicate that the Ad-E7- Δ Ct Δ TmCD40L vector is more efficient at inducing a cytokine release from the DCs than the Ad-E7-CD40L vector or the other

WO 2004/044176

PCT/US2003/036237

control vectors. In addition, since the secreted tumor antigen/CD40 ligand complex should engage the CD40 receptor on dendritic cells to effect cytokine release, these results suggest that the E7- Δ Ct Δ TmCD40L fusion protein forms a functional trimeric molecule at its carboxyl-terminal end to engage the CD40 receptor.

5 **6. Determination of cancer immunity developed by Adenoviral expression vectors**

[0071] To assess the efficacy of the Ad-E7- Δ Ct Δ TmCD40L vector for prevention of engraftment of the E7 positive TC-1 cell line in C57BL/6 mice, 1×10^8 pfu of each
10 vector was injected subcutaneously into mice. Seven days later, a repeat (boost) dose of the same vector was given. One week after the last vaccination, 5×10^5 TC-1 cells were injected subcutaneously on the back of the C57BL/6 mice at a site separate from the which vector injection site. Following Ad-E7- Δ Ct Δ TmCD40L vector injection, all of the mice were tumor free at day 108 after tumor challenge. In contrast, following
15 injection with Ad- Δ Ct Δ TmCD40L; Ad-CD40L; Ad-E7-CD40L, all (5/5) mice developed tumors within 15 days after tumor challenge, and all had died by day 42.

[0072] To characterize the types of effector cells involved in the suppression of tumor growth, mice were depleted of CD4 or CD8 T cells by antibody treatment prior to vaccination with Ad-E7- Δ Ct Δ TmCD40L. Antibody treatment continued even after the
20 TC-1 challenge to ensure proper depletion of the T cells subsets. It was observed that a TC-1 tumor grew in the mice that were depleted of either the CD4 or CD8 T cells. This suggests that presence of both CD4 and CD8 T cells are important for the antitumor effect induced by Ad-E7- Δ Ct Δ TmCD40L.

25 **7. DC binding of fusions proteins expressed by Adenoviral expression vectors.**

[0073] To test whether the secretable E7- Δ Ct Δ TmCD40L protein binds to DCs in the areas close to the site of vector injection, as predicted in the model in FIG. 1, the skin at the site of intradermal injection of the Ad-E7- Δ Ct Δ TmCD40L vector was sectioned and
30 double stained these sections with antibodies to the CD40L, and the DC markers CD80 and MHC-II. A widespread distribution of double-staining was observed in the epidermis from mice vaccinated with Ad-E7- Δ Ct Δ TmCD40L and Ad- Δ Ct Δ TmCD40L vectors. These results showed soluble E7- Δ Ct Δ TmCD40L protein binding to the

WO 2004/044176

PCT/US2003/036237

surface of DCs following injection of the Ad-E7- Δ Ct Δ TmCD40L. In contrast, only a few positive cells were observed in the epidermis following injection of the Ad-E7-CD40L, and the Ad-E7-CD40L vectors in the vaccinated mice at 3 days after the vector injection. These results indicate that the injection of the vector carrying the secretable form of the E7/CD40L is much more effective in generating protein that binds to the DCs than is the E7/CD40L protein that does not contain the signal peptide and has the transmembrane domain. The use of the vector carrying the E7/ Δ TmCD40L transcription unit which encodes a secreted form of the protein appears to amplify the effect of the vector from the infected DCs to a much larger population of DCs. The results suggest that the injection of the vector carrying the secretable form of the E7/CD40L protein will generate much higher levels of activated DCs loaded with the E7 than injections of vector carrying the non-secretable form of this protein. This prediction was tested directly in the next section.

8. Evaluation of DCs migration *in vitro* and *in vivo* induced by vaccination using Adenoviral Expression Vectors.

[0074] Following the binding of the CD40 ligand to the CD40 receptor, internalization of the tumor antigen/CD40 ligand-CD40 receptor complex and the subsequent digestion and processing of tumor antigen peptides, the DCs migrate to secondary lymphoid organs- regional lymph nodes to become mature DCs. In that location, the activated/antigen loaded DCs can present the tumor associated antigenic peptides and stimulate naïve CD4 helper T cells and amplify CD8 cytotoxic T cell lymphocytes (CTL) which are selectively toxic for cancer cells which carry the tumor associated antigen.

[0075] In order to test if the injection of AdE7- Δ Ct Δ TmCD40L vectors induced such a migration of the DCs to the regional lymph nodes *in vivo*, DCs that were derived *in vitro* from bone marrow cells were labeled by the supravital dye CFSA and then exposed to vector Ad-E7-Ct Δ TmCD40L, Ad-E7-CD40L, Ad- Δ Ct Δ TmCD40L and Ad-CD40L under conditions (100 MOI) designed to generate 100% infectivity. The infected dye loaded DCs were injected into the left flank of the C57BL/6 mice. Three days after these injections, the mice were sacrificed and the axillary lymph nodes on either side were harvested and studied for the presence of the dye loaded DCs. CFDA SE stained DC's carrying the secretable E7/CD40L transcription unit were observed in

WO 2004/044176

PCT/US2003/036237

the regional lymph nodes, whereas CFDA SE stained DC's carrying the E7/CD40L transcription unit were not observed in regional lymph nodes.

9. Evaluation of the immune response elicited following administration of Adenoviral expression vectors.

- 5 [0076] CD8+ T cell lymphocytes are involved in protective immune responses to infected or tumor cells. It was examined whether E7-specific CD8+ T cells were induced by subcutaneous injection of C57BL/6 mice with the Ad-E7- Δ Ct Δ TmCD40L vector. CD8+ T cell responses were assessed using cytotoxicity assays and the detection of antigen-specific CTLs using the enzyme-linked immunospot (ELISPOT) assay.
- 10 [0077] Mice were given two subcutaneous injections with the following vectors: (1) Ad-E7- Δ Ct Δ TmCD40L; (2) Ad-CD40L; (3) Ad-E7-CD40L; (4) Ad- Δ Ct Δ TmCD40L; and (5) negative control. Spleen cells from vector injected mice were stimulated by exposure to the TC-1 cells at a ratio of 25 TC-1 cells to one spleen cell for 48 hours. The restimulated spleen cells were then plated in 96 cell nitrocellulose filter plates which had
- 15 been coated with antibodies to either interferon gamma or IL-4. Following the washing of the wells with saline, the ELISPOT assay was carried out. The results of the ELISPOT indicated that the subcutaneous injection of the vector carrying the secretable E7/CD40L protein significantly increased MHC class I-restricted CTLs that were detectable with IFN- γ in spleens to a degree which was greater than that seen following
- 20 injection of the vector carrying the non-secretable E7/CD40L transcription unit or other control vectors. Also, The frequency of cells producing the T1-type cytokine IFN- γ (117 ± 10.61) was significantly higher than frequency of splenocytes capable of secreting a Th2 cytokine such as IL-4 (22.3 ± 3.68). These data indicate that the E7- Δ Ct Δ TmCD40L vector vaccination stimulates a Th1 rather than a Th2 immune
- 25 response.
- [0078] To determine if CD8 T cell lymphocyte effector cells specifically toxic for cancer cells bearing the E7 antigen were generated following injection of C57BL/6 mice following injection of the vector carrying the secretable E7/CD40L vector, splenocytes obtained animals given two subcutaneous injections of vector were incubated *in vitro*
- 30 with mitomycin C-treated TC-1 cells for 5 days for restimulation. Restimulated effector cells were mixed with TC-1 or control target cells for 4 hours and the release of LDH was measured. It was observed that spleen cells from mice injected with the Ad-E7-

WO 2004/044176

PCT/US2003/036237

Δ Tm CD40L vector lysed 90% of the TC-1 target cells. No apparent lysis was observed against irrelevant but syngeneic EL-4 cells, which do not carry the E7 antigen. The level of cytotoxicity to TC-1 cells was higher with splenocytes taken from the Ad-E7- Δ Ct Δ TmCD40L vector inject mice than with splenocytes taken from mice previously

5 injected with the Ad-E7-CD40L vector.

9. Materials and Methods

a) Mice

[0079] Six- to 8-wk-old C57BL/6 mice were purchased from Harlan.

b) Cell lines

10 [0080] The C57BL/6 syngeneic TC-1 tumor was immortalized with the HPV-16 E6 and E7 genes and transformed with the c-Ha-*ras* oncogene (25). TC-1 expresses low levels of E6 and E7 and is highly tumorigenic. TC-1 was grown in RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 100 μ M nonessential amino acids, 1 mM sodium pyruvate, 50 μ M 2-ME, at 37° with 10% CO₂.

15 c) Production of recombinant Adenoviruses

[0081] A transcription unit that included DNA encoding the signal peptide from the HGH gene upstream of DNA encoding the full length HPV type 16 E7 protein upstream of Δ Ct Δ TmCD40L was generated. DNA encoding the human growth hormone signal sequence MATGSRTSLLLAFLGLCLPWLQEGSA (single letter amino acid code)

20 (SEQ ID NO: 1) was prepared by annealing phosphorylated oligonucleotides (SEQ ID NOs:2 and 3) to generate the full 26 amino acid HGH sequence with Bgl II and NotI overhangs.

Growth hormone signal upper strand (coding sequence in italics):

5'-GATCT CCACC *ATG GCT ACA GGC TCC CGG ACG TCC CTG CTC CTG GCT*

25 *TTT GGC CTG CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT GCC GGC* -3'

(SEQ ID NO: 2)

WO 2004/044176

PCT/US2003/036237

Growth hormone signal lower strand:

3'-A GGTGG TAC CGA TGT CCG AGG GCC TGC AGG GAC GAG GAC CGA
AAA CCG GAC GAG ACG GAC GGG ACC GAA GTT CTC CCG TCA CGG
CCGCCGG -5'. (SEQ ID NO:3)

5

[0082] Synthetic HGH signal sequence was prepared by annealing the above upper and lower strand oligos. The oligos were dissolved in 50 μ l H₂O (about 3 mg/ml). 1 μ l from each oligo (upper and lower strand) was added to 48 μ l annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2 mM Mg-acetate) incubated at 4
10 minutes at 95°C, 10 minutes at 70°C and slowly cooled to about 4°C. The annealed DNA was phosphorylated using T4 PNK (polynucleotide kinase) under standard conditions.

[0083] The HGH signal sequence with Bgl II and Not I overhangs was inserted via Bgl II and Not I into pShuttle-E7- Δ Ct Δ TmCD40L(no signal sequence) to yield pshuttle-
15 HGH/E7- Δ Ct Δ TmCD40L.

[0084] pShuttle-E7- Δ Ct Δ TmCD40L (no signal sequence) was prepared as follows: Plasmid pDC406-mCD40L was purchased from the American Type Culture Collection. A pair of PCR primers (SEQ ID NOs: 4 and 5) was designed to amplify the mouse
20 CD40 ligand from position 52 to 260 (i.e., without the cytoplasmic and transmembrane domains) and include sequence encoding a linker (indicated as "+ spacer") at the 5' end of the amplicon.

Mouse Δ Ct Δ TmCD40L+ spacer forward primer (MCD40LSPF) (CD40L sequence italicized):

5'- CCG CTCGAG AACGACGCACAAGCACCAAAATCAAAGGTCGAAGAGGAA
25 GTA -3' (SEQ ID NO: 4).

Mouse CD40L reverse primer (MCD40LR)

5'-CCC AAGCTT ATCAGAGTTTCACTAAGCCAA-3' (SEQ ID NO: 5)

[0085] The forward primer MCD40LSPF encoded a 10 residue spacer (FENDAQAPKS; single letter code; SEQ ID NO: 6) to be located between the tumor

WO 2004/044176

PCT/US2003/036237

antigen and the CD40 ligand (mCD40L) of the transcription unit. PCR performed using the forward and reverse primers (SEQ ID NOs 4 and 5) and Plasmid pDC406-mCD40L as the template resulted in PCR fragment "space+ΔCtΔTMCD40L", which was inserted into the plasmid pShuttle-CMV (13) after restriction endonuclease digestion with Hind III (AAGCTT) and Xho I (CTCGAG). This vector is designated pShuttleΔCtΔTmCD40L. A vector was produced that was otherwise the same except that it encoded full length CD40L rather than the truncated form. This vector was made using a CD40 forward primer that annealed to the starting codons of murine CD40L. This vector is designated pShuttleCD40L (no signal sequence).

10 [0086] Modification of pShuttleΔCtΔTmCD40L(no signal sequence) to include the HPV-16 E7 upstream of the CD40 ligand sequence was accomplished as follows: Sequence encoding the full HPV-16 E7 protein was obtained by PCR amplifying from the HPV viral genome using the following primers:

HPV 16 E 7 forward primer (SEQ ID NO: 7)

15 5'-ATTT GCGGCCGC TGTAATCATGCATGGAGA-3'

HPV E7 reverse primer (SEQ ID NO: 8)

5-CC CTCGAG TTATGGTTTCTGAGAACAGAT-3'

The resulting amplicon was HPV 16 E 7 encoding DNA with 5' end Not I and 3' end Xho I restriction sites. The E7 DNA was inserted into the pShuttleΔCtΔTmCD40L between the CMV promoter and directly 5' to the spacer of the ΔCtΔTMCD40L sequence using Not I (GCGGCCGC) and Xho I (CTCGAG). The plasmid is designated pShuttle-E7-ΔCtΔTmCD40L (no signal sequence) and was used for insertion of the HGH signal sequence upstream of E7 to generate HGH/E7-ΔCtΔTmCD40L as already described. Thus, the transcription unit HGH/E7-ΔCtΔTmCD40L encodes the HGH secretory signal followed by the full length HPV type 16 E7 followed by a 10 amino acid linker with (FENDAQAPKS; SEQ ID NO: 9) followed by murine CD40 ligand residues 52-260.

[0087] A similar procedure as described above was used to insert the E7 encoding DNA upstream of full length CD40 in pShuttleCD40L(no signal sequence).

WO 2004/044176

PCT/US2003/036237

[0088] In a similar fashion, the green fluorescent protein (GFP) gene was inserted downstream of the CMV promoter and upstream the mΔCtΔTmCD40L or mCD40L (wildtype CD40L) and in a replication incompetent adenoviral vector. These vectors were used as controls. Other adenoviral vectors were created by inserting DNA
 5 encoding the HGH signal sequence between the CMV promoter and 5' to DNA encoding ΔCtΔTmCD40L or CD40L of vector pShuttle-ΔCtΔTmCD40L or pShuttle-CD40L, respectively, to create vectors pShuttle-HGHΔCtΔTmCD40L and pShuttle-HGHCD40L, respectively.

[0089] A transcription unit that included DNA encoding the signal sequence of the
 10 mouse IgG kappa chain gene upstream of DNA encoding the full length HPV type 16 E7 protein ("K/E7") was generated by PCR using HPV16 plasmid and the following primers:

5'-ACG ATG GAG ACA GAC ACA ~~CTC CTG CTA TGG GTA CTG CTG~~-3'
 (SEQ ID NO: 10)

15

5'- TC CTG CTA TGG GTA ~~CTG CTG CTC TGG GTT CCA GGT TC~~-3'
 (SEQ ID NO: 11)

20

5'- ~~TC CTC TGG GTT CCA GGT TCC~~ ACT GGT GAC ATG CAT G-3'
 (SEQ ID NO: 12);

5'- TGG GTT CCA GGT TCC ACT GGT GAC ATG CAT GGA G AT ACA CCT
 AC-3' (SEQ ID NO: 13); and

25

5'- CCG CTC GAG TGG TTT CTG AGA ACA GAT GGG GCA C -3.'
 (SEQ ID NO: 14)

K/E7 with the upstream kappa signal sequence was generated by four rounds of PCR
 amplification (1st round: primers 4 +5; 2nd round: add primer 3; 3rd round: add primer 2;
 30 4th round: add primer 1). The K/E7 encoding DNA was cloned into the pcDNA™ 3.1
 TOPO vector (Invitrogen, San Diego, CA) forming pcDNA-K/E7.

WO 2004/044176

PCT/US2003/036237

[0090] A DNA fragment that contained the mouse CD40 ligand from which the transmembrane and cytoplasmic domain had been deleted (Δ Ct Δ TmCD40L) was generated from a mouse CD40 ligand cDNA Plasmid (pDC406-mCD40L; ATCC) using the following PCR primers:

5 5'-CCG CTCGAG AAC GAC GCA CAA GCA CCA AAA AGC AAG GTC GAA GAG
GAA GTA AAC CTT C-3' (SEQ ID NO: 15); and

5'-CGCGCCGCGCGCTAG TCTAGA GAGTTTGAGTAAGCCAAAAGATGAG-
3' (SEQ ID NO: 16) (high fidelity PCR kit, Roche).

10

Fragment Δ Ct Δ TmCD40L was digested with Xba I and XhoI restriction endonucleases and then ligated into pcDNA-E7. K/E7- Δ Ct Δ TmCD40L fragment was cut from the pcDNA vector and inserted into the pShuttle plasmid using Hind III and Xba I sites (pShuttle K/E7-Ct Δ TmCD40L). Thus, the K/E7- Δ Ct Δ TmCD40L fragment includes the
15 kappa chain secretory signal followed by the full length HPV type 16 E7 followed by a 10 amino acid linker (LQNDAQAPKS; SEQ ID NO: 17) followed by murine CD40 ligand residues 52-260.

[0091] Adenoviral vector encoding a fusion protein with E7 upstream of full length mouse CD40L was made using primers to amplify full length mouse CD40L. The
20 following primers were used:

5'- GAGAC CTC GAG CAGTCA GC ATGATAGA AACATACAGC
CAACCTTCCC-3' (SEQ ID NO: 18);

25 5'- CCGCGC CCCAAGCTTA TCAGAGTTTGAGTAAGCCAAAAG-3'. (SEQ ID NO: 19).

Amplified DNA was initially subcloned into the pCDNA3 with Xba I and XhoI restriction endonucleases. The full length CD40L gene or Δ Ct Δ TmCD40L was
30 directionally cloned into the pShuttle plasmid with the Hind III and Xba I sites. An overview of the scheme for constructing adenoviral expression vector encoding E7- Δ Ct Δ TmCD40L is shown in FIG. 2.

WO 2004/044176

PCT/US2003/036237

[0092] The recombinant adenoviral vectors were generated using the AdEasy vector system (Stratagene, San Diego, CA). Briefly the resulting plasmid pShuttle-HGH/E7- Δ Ct Δ TmCD40L, pShuttle-HGH/CD40L, pShuttle-HGH/E7-CD40L, pShuttle-HGH/GFP, pShuttleHGH/GFP-CD40L, and pShuttle-HGH/E7 were linearized with *Pme* I and co-transformed into *E. coli* strain BJ5183 together with pAdEasy-1, the viral DNA plasmid. Recombinants were selected with kanamycin and screened by restriction enzyme analysis. The recombinant adenoviral construct was then cleaved with *Pac* I to expose its Inverted Terminal Repeats (ITR) and transfected into 293A cells to produce viral particles. The titer of recombinant adenovirus was decided by Tissue culture Infectious Dose (TCID₅₀) method.

[0093] Primers for amplifying human Δ Ct Δ TMCD40L+ spacer using a human CD40 ligand cDNA template are set forth below.

Human Δ Ct Δ TmCD40L+ spacer forward primer (HCD40LSPF) (CD40L sequence italicized):

5'- CCG

CTCGAGAACGACGCACAAGCACCAAAATCACATAGAAGGTTGGACAA

G-3' (SEQ ID NO: 20).

Human CD40L reverse primer (HCD40LR)

20 5'-CCC AAGCTT TCAGAGTTTGAGTAAGCCAAAGGAC-3' (SEQ ID NO: 21)

These primers will amplify a Δ Ct Δ TmCD40L+spacer which encodes 47-261 of human CD40L. The forward primer HCD40LSPF encodes a 10 residue spacer (FENDAQAPKS; single letter code; SEQ ID NO: 9) to be located between the tumor antigen and the CD40 ligand (hCD40L) of the transcription unit. PCR performed using the forward and reverse primers (SEQ ID NOs 20 and 21) and Plasmid pDC406-hCD40L as the template results in PCR fragment "space+ Δ Ct Δ TmCD40L(human)", which is inserted into the plasmid pShuttle-CMV (13) after restriction endonuclease digestion with Hind III (AAGCTT) and Xho I (CTCGAG). This vector is designated pShuttle Δ Ct Δ TmCD40L(human). Modification of pShuttle Δ Ct Δ TmCD40L(human) to

WO 2004/044176

PCT/US2003/036237

include the HPV-16 E7 upstream of the human CD40 ligand sequence was accomplished essentially as described above for the murine CD40 ligand encoding vectors. The resulting plasmid is designated pShuttle-E7- Δ Ct Δ TmCD40L(human)(no signal sequence) and is used for insertion of the HGH signal sequence upstream of E7 to
5 generate HGH/E7- Δ Ct Δ TmCD40L(human). Thus, the transcription unit HGH/E7- Δ Ct Δ TmCD40L(human) encodes the HGH secretory signal followed by the full length HPV type 16 E7 followed by a 10 amino acid linker (FENDAQAQPKS; SEQ ID NO:9) followed by human CD40 ligand residues 47-261.

10 **d) *In vitro* expression of Adenoviral vector transcription units**

[0094] The coupled *in vitro* transcription-translation system of RILL (TNT kits from Protégé Corp.) was used for the synthesis of the following vectors: Ad-E7-Ct Δ TmCD40L, Ad-E7-wtTmCD40L, Ad- Δ Ct Δ TmCD40L, Ad-wtTmCD40L, and Ad-E7. Two μ g of each plasmid DNA or PCR generated template DNA was added to the 50
15 μ l reaction mixture containing 25 μ l of RRL, 2 μ l of TNT reaction buffer, 1 μ l of T7 RNA polymerase, 1 μ l of amino acid mixture (1mM) without methionine, 4 μ l of [35 S]-methionine, and 1 μ l of ribonuclease inhibitor RNAsin (40U/ μ l). The reaction was completed by incubation at 30 °C for 1 hr. 15 μ l of each [35 S]-labeled product was run on a 10% SDS-PAGE gel and exposed to X-film overnight.

20 **e) Western blot Analysis**

[0095] The cell lysate derived from 293 cells infected by an adenoviral vector at MOI 40 was fractionated on a 10% reducing SDS-PAGE gel and transferred to an IMMOBILON-P membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk for 2 h at room temperature, the membrane was probed with an antibody against
25 the specific mouse CD40 ligand (mCD40L) in TBS-T buffer (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, and 0.5% Tween 20) in the presence of 2% BSA overnight. After washing four times with TBS-T buffer, the blot was incubated with goat anti-hamster alkaline phosphatase conjugated antibody (Jackson Immunoresearch) for 1 hour. The immunoreactive bands were visualized on membrane by using the ProtoBlot II AP
30 system (Promega Corp.).

f) Flow cytometry analyses of DC

WO 2004/044176

PCT/US2003/036237

[0096] To quantify the expression level of surface molecules of APCs or DCs, the test cells were stained with FITC- or PE-conjugated anti-mouse monoclonal antibodies (mAbs) for CD80, CD54, and CD11c (Pharmingen), for 30 min on ice, prior to immunostaining with labeled Abs. The APCs were first incubated with a Fc- γ blocking antibody (anti-mouse CD16/CD32 antibody) to avoid the nonspecific binding of mAbs to Fc- γ receptors. The cells were then washed twice, fixed in 4% paraformaldehyde, and analyzed using a Becton Dickinson flow cytometer (FACS Calipur).

g) Cytokine production of Ad-mCD40L-modified DCs

[0097] The DCs were infected with the following adenoviral vectors: Ad E7- Δ Ct Δ TmCD40L, AdmCD40L, AdE7-CD40L, AdGFP, AdGFP-mCD40L, AdE7 or phosphate-buffered saline (PBS) at MOI 100, and plated in 24-well plates at 2×10^5 cells/ml. After incubation for 24 hours at 37°C, the supernatant fluid (1ml) was harvested and centrifuged to remove debris. The level of murine IL-12 or IFN- γ released into the culture medium was assessed by enzyme-linked immunosorbent assay (ELISA), using the mouse IL-12 p70 or IFN- γ R & D Systems respectively.

h) DC migration Assays

[0098] The protocol used to study the *in vitro* migration of DCs was the same as that used by Romani et al (21). In brief, the skin of the ear of mice was split in dorsal and ventral halves and dorsal halves were cultured on the bottom membrane of the inner chamber of six well double-chamber tissue culture plate (transwell no 3414, Costar). The outer chamber was loaded with 1×10^8 vector particles. It was then filled with culture medium so that the vector particles could bath the skin from underneath. The epidermal part of the skin was thus exposed to air. The cultures were incubated at 37°C for 3 days. The number of DCs that had emigrated from the skin into culture medium was counted under the hemocytometer.

[0099] The DC *in vivo* migration experiment was carried out as follows. After the bone marrow derived DCs were cultured 6 days with IL-3 ,GM-CSF, they were loaded with the CFDA SE suprevital dye. Briefly, the DCs were incubated with 10 ul of CFDA SE for 15 min at 37 °C. The dendritic cells were then pelleted and resuspended in fresh prewarmed medium for 30 min. The cells were mixed with each recombinant adenoviral vector at MOI 200. The vector was then injected into the left flank of the test mouse. Three days later, the axillary lymph nodes draining the region of the injection

WO 2004/044176

PCT/US2003/036237

site for the DCs were removed, frozen tissue sections made and observed in the immunofluorescence microscope.

i) Immunohistochemical Staining

Vaccinated mice were sacrificed 3 days after the Ad E7- Δ Ct Δ TmCD40L, Ad-
5 Δ Ct Δ TmCD40L, AdE7-CD40L, AdE7-CD40L vector vaccination. The skin at the site
of vector subcutaneous injection was biopsied, embedded in Oct solution, and cut into 5-
 μ m sections. The slides were incubated with rat anti-MHC-II antibody, rat anti-CD40L
antibody, and rat anti-CD80 polyclonal Ab (Bioscience). This was followed by exposure
to biotinylated goat anti-rat IgG antibody (1:200 dilution) and avidin-biotin complex
10 (1:100 dilution; Vector, Burlingame, CA). The stained slides then were mounted, and
studied under a fluorescence microscope.

j) Cytokine Profile by ELISPOT Assays

[00100] The presence of E7-specific effector T cells in the immunized mice was also
assessed in ELISPOT assays, as previously described (26,27). Briefly, splenocytes
15 obtained from mice vaccinated with each of the different vectors were restimulated *in*
vitro by culture with the TC-1 cell line (responder-to-stimulator ratio 25:1) in the
presence of 10 U/ml IL-2 for 48 hours. Re-stimulated splenocytes were then plated in
96-well nitrocellulose filter plates (5×10^4 cells in 100 microliters). The wells were pre-
coated with rat anti-mouse anti-IFN-antibody or anti-IL-4 antibody. After incubation
20 for 24 hours at 37°C/5% CO₂, the plates were then washed with PBS, and the presence
of cytokine-producing spleen cells was detected by incubation at 4°C with biotinylated
goat anti-rat secondary antibody, followed by 100 microliter/well horseradish
peroxidase avidinD and 150 microliter/well freshly prepared substrate buffer (0.4 mg/ml
3-amino-9-ethyl-carbazole in a total of 50 ml 0.05 mol/L sodium acetate buffer) and 20
25 microliter 30% H₂O₂. The stained spots corresponding with IFN producing cells or IL-4
producing cells were enumerated under a dissecting microscope.

WO 2004/044176

PCT/US2003/036237

k) Cytotoxicity Assay

[00101] For these studies, mice received vaccination via a subcutaneous injection of the following vectors: Ad-E7- Δ Ct Δ TmCD40L (secretable CD40 ligand), Ad- Δ Ct Δ TmCD40L (secretable CD40 ligand), Ad-E7-CD40L (non-secretable CD40L), Ad-CD40L (non-secretable ligand) and Ad-E7. The vectors were injected on days 0 and 7 as a subcutaneous injection. As a control, PBS was injected subcutaneously on days 0 and 7 (control mice). Mononuclear cells from the spleens of these mice (vector injected or PBS injected) were used as the source of effector cells for cytotoxicity studies. Aliquots of the spleen cell suspensions were then co-incubated with mitomycin C-treated TC-1 cells in RPMI 1640 medium, supplemented with 10% FBS, 50 M 2-mercaptoethanol, 2 mM glutamine, 1mM pyruvate, and nonessential amino acids, under cell culture conditions for 5 days. To perform the cytotoxicity assay, 5×10^3 of TC-1 tumor cells (target cells) were incubated with the stimulated splenic mononuclear cells (effector cells) at an effector/target ratio of 100:1 for 4 hours at 37°C, in culture media containing 5% FBS. At the end of the incubation, mononuclear cell-mediated cytotoxicity was determined using the nonradioactive cytotoxicity assay kit. Released LDH in culture supernatants was measured by an ELISA plate reader. The data were calculated as:

% CYTOTOXICITY = $(\text{EXR ESR TSR} + \text{CMB/TMR TSR VC} + \text{CMB}) / 100$ where EXR is experimental LDH release, ESR is effector cell spontaneous LDH release, TSR is target cell spontaneous LDH release, TMR is target cell maximum LDH release, VC is volume correction, and CMB is culture medium LDH back-ground. Statistics: Student's unpaired t-test was used to determine the differences between the various groups in the proliferation and cytotoxicity assays. Statistical significance was determined at the 0.01 level.

l) *In vivo* Efficacy Experiment in Mouse Model

[00102] Mice (5 or 10 per group) were vaccinated via subcutaneous injection with 1×10^8 PFU Ad-E7- Δ Ct Δ TmCD40L, Ad-E7, Ad-E7-CD40L, Ad- Δ Ct Δ TmCD40L or Ad-CD40L vectors. One week later, mice were boosted with the same adenoviral vector regimen as the first vaccination. One week after the last vaccination, mice were challenged by subcutaneous injection of 5×10^5 TC-1 cells/mouse in the right leg and then monitored twice a week.

WO 2004/044176

PCT/US2003/036237

[00103] The donor splenocytes were harvested from tumor-free donor C57BL/6 mice and passed over magnetic columns to enrich for total T cells. Tumor growth was measured as described previously.

m) Statistics

- 5 [00104] All parameters were analyzed using Student's *t* test, or ANOVA followed by Scheffé's procedure for multiple comparisons as *post-hoc* analysis; all data shown is presented as mean \pm S.E. of the mean (S.E.).

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WO 2004/044176

PCT/US2003/036237

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WO 2004/044176

PCT/US2003/036237

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WO 2004/044176

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5 [00105] All patents and publications mentioned in the specification are indicative of
the levels of those of ordinary skill in the art to which the invention pertains. All patents
and publications are herein incorporated by reference to the same extent as if each
individual publication was specifically and individually indicated to be incorporated by
reference.

10 [00106] The invention illustratively described herein suitably may be practiced in the
absence of any element or elements, limitation or limitations which is not specifically
disclosed herein. Thus, for example, in each instance herein any of the terms
“comprising,” “consisting essentially of” and “consisting of” may be replaced with
either of the other two terms. The terms and expressions which have been employed are
used as terms of description and not of limitation, and there is no intention that in the
15 use of such terms and expressions of excluding any equivalents of the features shown
and described or portions thereof, but it is recognized that various modifications are
possible within the scope of the invention claimed. Thus, it should be understood that
although the present invention has been specifically disclosed by preferred embodiments
and optional features, modification and variation of the concepts herein disclosed may
20 be resorted to by those skilled in the art, and that such modifications and variations are
considered to be within the scope of this invention as defined by the appended claims.

[00107] Other embodiments are set forth within the following claims.

WO 2004/044176

PCT/US2003/036237

We claim:

1. An adenoviral expression vector for generating immunity against a tumor antigen, said vector comprising a transcription unit encoding a polypeptide, said polypeptide comprising from the amino terminus a secretory signal sequence, a tumor antigen, and CD40 ligand, wherein said tumor antigen is different from CD40 ligand and wherein said CD40 ligand is missing all or substantially all of the transmembrane domain rendering the CD40L secretable.
2. The adenoviral expression vector of claim 1 wherein said tumor antigen is a human tumor antigen.
3. The adenoviral expression vector of claim 2 wherein said tumor antigen is the E7 protein of human papilloma virus.
4. The adenoviral expression vector of claim 1 wherein said transcription unit encodes a linker between said tumor antigen and said CD40 ligand.
5. The adenoviral expression vector of claim 1 wherein said vector includes a human cytomegalovirus promoter/enhancer for controlling transcription of the transcription unit.
6. The adenoviral expression vector of claim 1 wherein said CD40 ligand is human CD40 ligand.
7. The adenoviral expression vector of claim 1 wherein said CD40 ligand lacks a cytoplasmic domain.
8. The adenoviral expression vector of claim 1 wherein the vector encodes a CD40L that includes no more than six residues from either end of the transmembrane domain.
9. The adenoviral expression vector of claim 1 wherein said vector does not encode the transmembrane domain of CD40 ligand.
10. The adenoviral expression vector of claim 6 wherein said CD40 ligand comprises residues 47-261.

WO 2004/044176

PCT/US2003/036237

11. The adenoviral expression vector of claim 6 wherein said CD40 ligand comprises residues 1-23 and 47-261.
12. The adenoviral expression vector of claim 1 wherein said vector is rendered non-replicating in normal human cells.
- 5 13. A method of generating an immune response in an individual against cells expressing a tumor antigen, comprising administering to the individual an effective amount of the adenoviral expression vector of claim 1.
14. The method of claim 13 wherein said tumor antigen is the E7 protein of human papilloma virus.
- 10 15. The method of claim 13 wherein said CD40 ligand is human CD40 ligand.
16. The method of claim 13 wherein said cancer cells are cervical cancer cells.
17. The method of claim 16 wherein said tumor antigen is E7 protein of human papilloma virus.
18. The method of claim 13 wherein said administering is repeated.
- 15 19. The method of claim 13 wherein said immune response includes the generation of cytotoxic CD8⁺ T cells against said tumor associated antigen.
20. The method of claim 13 wherein following administration, said vector is taken up by cells which subsequently secrete a fusion protein encoded by the transcription unit.
- 20 21. The method of claim 20 wherein said fusion protein forms a homotrimer through interaction of CD40 ligand extracellular domains.
22. A method of treating an individual with cancer that expresses a tumor antigen, comprising administering to the individual an effective amount of the adenoviral expression vector of claim 1.

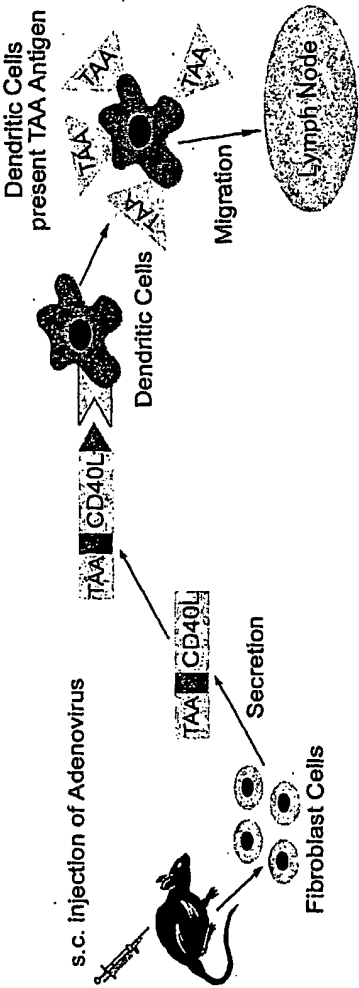
WO 2004/044176

PCT/US2003/036237

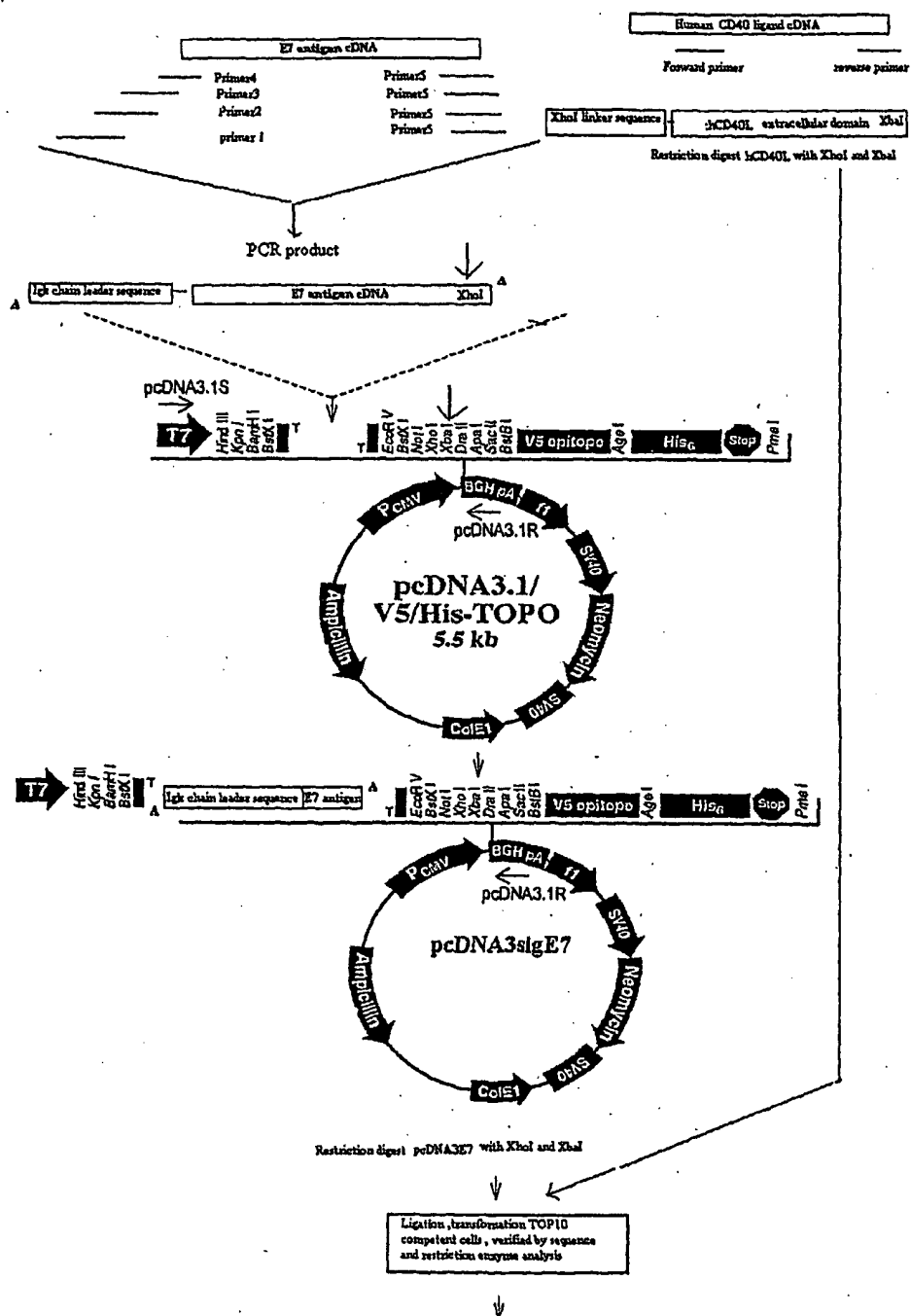
23. The method of claim 22 wherein said tumor antigen is the E7 protein of human papilloma virus.
24. The method of claim 22 wherein said CD40 ligand is human CD40 ligand.
25. The method of claim 22 wherein said cancer is cervical cancer.
- 5 26. The method of claim 25 wherein said tumor antigen is E7 protein of human papilloma virus.
27. The method of claim 22 wherein said administering is repeated.
28. The method of claim 22 wherein said immune response includes the generation of cytotoxic CD8⁺ T cells against said tumor associated antigen.
- 10 29. The method of claim 22 wherein following administration, said vector is taken up by cells which subsequently secrete a fusion protein encoded by the transcription unit.
30. The method of claim 29 wherein said fusion protein forms a homotrimer through interaction of CD40 ligand extracellular domains.
- 15 31. A method of generating immunity in a subject to infection by human papilloma virus, comprising administering to the individual an effective amount of the adenoviral expression vector of claim 1 wherein said tumor antigen is the E6 or E7 protein of human papilloma virus.
32. The method of claim 31 wherein said CD40 ligand is human CD40 ligand.
- 20 33. The method of claim 31 wherein said administering is repeated.
34. The method of claim 31 wherein said immune response includes the generation of cytotoxic CD8⁺ T cells against human papilloma virus.

1/3

FIG. 1



2/3



[illegible]

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